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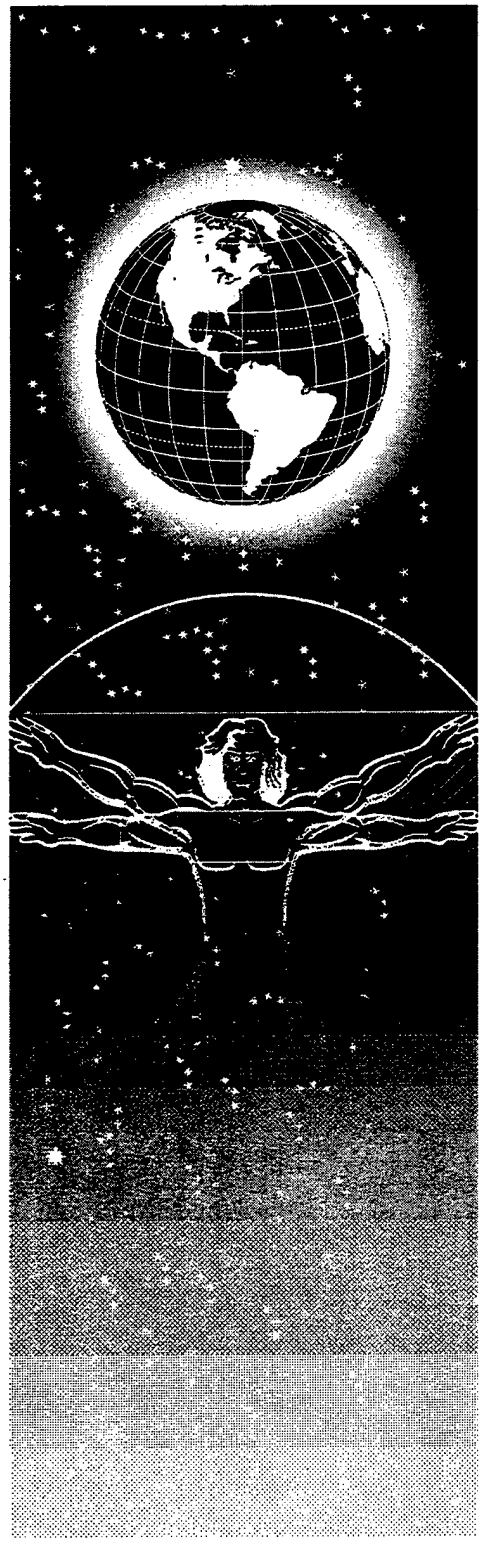

# UNITED STATES AIR FORCE RESEARCH LABORATORY

## A PILOT STUDY OF OCCUPATIONAL ASSESSMENT OF AIR FORCE PERSONNEL EXPOSURE TO JET FUEL BEFORE AND AFTER CONVERSION TO JP-8

**Donna M. Olsen**
UNIVERSITY OF CINCINNATI
HILL AIR FORCE BASE, UT

**David R. Mattie**
OPERATIONAL TOXICOLOGY BRANCH
2856 G STREET, BLDG 79
WRIGHT-PATTERSON AFB OH 45433-7400

**William D. Gould**
DET 1, HSC/OEM
BROOKS AFB, TX

**Frank Witzmann**
MOLLECULAR ANATOMY LABORATORY
INDIANA UNIVERSITY - PURDUE UNIVERSITY
COLUMBUS, IN

**Mark Ledbetter**
THE PSYCHOLOGICAL CORPORATION
SAN ANTONIO, TX

**Grace K. Lemasters**
DIVISION OF EPIDEMIOLOGY AND BIOSTATISTICS UNIVERSITY OF CINCINNATI
CINCINNATI, OH

**James H. Yin**
DEPARTMENT OF ENVIRONMENTAL HEALTH
UNIVERSITY CINCINNATI
CINCINNATI, OH

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**Human Effectiveness Directorate**
**Crew Survivability and Logistics Division**
**2856 G Street**
**Wright-Patterson AFB OH 45433-7400**

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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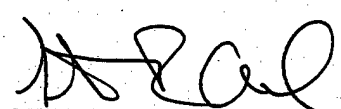

**STEPHEN R. CHANNEL,** Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Research Laboratory

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The purpose of this study was to compare workers' health while JP-4 was still in use and after conversion to JP-8. Measures of effect were blood tests for liver, kidney and hematopoietic system function and serum protein analysis, neurocognitive function, and general health (history and physical exam). A cohort of 18 exposed and 18 non-exposed subjects, matched for gender and age, were measured 4 times over a period of 18 months while JP-4 was used, and at 3, 6 and 18 months after conversion to JP-8. Air sampling showed levels of naphthas during JP-8 use comparable to JP-4. Benzene, non-detectable in JP-8, compared to 0.05 ppm in JP-4. No significant differences were found between exposed and non-exposed subjects in liver and kidney function, neurocognitive function, frequency of symptoms, or general health. A rash on the hands was observed in 2 subjects using JP-8. No changes were seen in any of the complete blood count parameters between JP-4 and JP-8 use. Mean Corpuscular Hemoglobin and Mean Corpuscular Volume were statistically significantly lower in the exposed subjects at all sampling periods, indicating it may be an effect of jet fuel exposure in general. Mean Corpuscular Hemoglobin Concentration was significantly higher after conversion to JP-8 in the exposed subjects. Further study of larger samples is needed.

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## ABBREVIATIONS

| | |
|---|---|
| AFB | Air Force Base |
| AFOSR | Air Force Office of Scientific Research |
| AFRL | Air Force Research Laboratory |
| ALT | Alanine transferase |
| APP | Acute Phase Proteins |
| AST | Aspartamine transferase |
| BUN | Blood urea nitrogen |
| C | Carbon |
| °C | Degrees centigrade |
| CIH | Certified Industrial Hygienist |
| CV | Coefficient of variation |
| 2-DE | Two dimensional gel electrophoresis |
| DoD | Department of Defense |
| EPA | Environmental Protection Agency |
| F-344 | Fischer-344 (rat) |
| GC-MSD | Gas chromatograph-mass spectrophotometer |
| Hg | Mercury |
| IEF | Isoelectric focusing |
| IH | Industrial hygiene |
| LEL | Lower explosive limit |

| | |
|---|---|
| m | Meter |
| ml | Milliliter |
| MCH | Mean corpuscular hemoglobin |
| MCHC | Mean corpuscular hemoglobin concentration |
| MCV | Mean corpuscular volume |
| mg | Milligram |
| n | Sample size |
| NIEHS | National Institute for Environmental Health Sciences |
| NIOSH | National Institute for Occupational Safety and Health |
| OSHA | Occupational Safety and Health Act |
| PAH | Polynuclear aromatic hydrocarbons |
| PEL | Permissible exposure limit |
| ppm | Parts per million |
| SD | Standard deviation |

# I. INTRODUCTION

The United States Air Force and most of the Department of Defense (DoD) have converted to a single aircraft fuel, JP-8. The Air Force now flies solely with JP-8 jet fuel for most of its aircraft and JP-8 is also used in some ground equipment. The Navy uses JP-8 at Naval air stations and a narrower distillate cut of JP-8 called JP-5 is used on board aircraft carriers and for aircraft flying to carriers. JP-5 has a higher flash point than JP-8 and meets all of the government specifications for JP-8. The Army is using JP-8 in some weapon systems and battle tanks and other ground support vehicles that previously ran on diesel fuel. The last region in the United States to convert from JP-4 to JP-8 consisted of Utah, Idaho, Montana, Colorado, and Wyoming. This region began conversion in October 1995.

The Air Force replaced JP-4 with JP-8 as aircraft fuel for economic, as well as health and safety reasons. Initially the Air Force used JP-8 in Europe because it was the only fuel available. Maintaining a single fuel for aircraft and ground equipment from all branches of the service would be more cost-effective, particularly in a battlefield setting. As to health and safety concerns, JP-4 contained benzene, an A1 carcinogen, which is present in JP-8 in much lower concentrations. It was thought that, since JP-4 was more volatile than JP-8, the use of JP-8 would lead to lower worker airborne or vapor exposure when using JP-8. Since the flash point of JP-8 is higher than JP-4, it would reduce the possibility of explosion during routine and combat operations.

Since billions of gallons of jet fuel are used by the DoD each year, thousands of military and civilian personnel are exposed to it. A study of the health of personnel exposed to jet fuel was needed since there were a number of worker health complaints soon after conversion to JP-8 jet fuel use.

## Background

There is limited human epidemiological data available for JP-4 and JP-8 jet fuels. Reports have been received involving eye, skin, and respiratory irritation symptoms in Air Force workers. JP-8 has been particularly troubling to the worker's skin with many workers complaining that JP-8 burns their skin and there have been reports of minor chemical burns. There has also been a question of liver toxicity with JP-8. There is limited human data regarding the effect of JP-8 on the hematopoietic system. The neurotoxicant n-hexane, which was present in JP-4, does not appear to be present in JP-8, or is at extremely low concentrations. There have been no reports of peripheral neurotoxicity with JP-8 thus far. However, there have been reports of headache and nausea which are non-specific but could represent central nervous system (CNS) depressant effects.

Although the level of benzene in JP-8 is less than in JP-4, there is some concern about aromatic compounds, such as benzene, accumulating in the foam lining of the fuel cells of certain aircraft (e.g. C-130 model). Another concern is the possibility of increased absorption of benzene through contact with skin and clothing due to the fact that JP-8 evaporates more slowly due to its lower vapor pressure (1.80 mm Hg at 28°C) and higher boiling point range (175-300 °C). The composition of JP-8 by volume percent is: $C_8$-$C_9$ aliphatic hydrocarbons (9%); $C_{10}$-$C_{14}$ aliphatic hydrocarbons (65%); $C_{15}$-$C_{17}$ aliphatic hydrocarbons (7%); and aromatics (18%).

The few studies of jet fuel toxicity published to date attribute the observed effects to the components of the fuel such as benzene, toluene, xylene, and n-hexane. Studies by Struwe *et al.*[1] and Knave *et al.*[2,3] have documented the occurrence of symptoms of neurasthenia, psychasthenia and polyneuropathy in civilian and military aircraft workers. They reported cases of sexual dysfunction which were possibly neurologic in origin. Reviews of the literature summarizing the health effects of jet fuel exposure have been published.[4,5] The major findings have been skin irritation and defatting, neurotoxicity, nephrotoxicity and renal carcinogenicity in male rats. Male mice showed significant testicular atrophy in a twelve month intermittent JP-4 inhalation study.[6] Limited information is available on the reproductive effects of JP-4 and JP-8 jet fuels in humans and animals. The results of a study of male reproductive effects by the University of Cincinnati will be published later this year.

Mutagenicity studies of commercial jet fuel (Jet A, which is similar in primary composition to JP-8) were negative for the Ames Salmonella test but were positive in mouse lymphoma, sister chromatid exchange and rat bone marrow cytogenetic assays.[7,8] In a study of JP-4 mutagenicity, evidence for pre-implantation loss was observed at mating 4 in a rat dominant lethal assay study by Brusick and Matheson.[9] Genetic toxicity tests, which included the Ames Salmonella, mouse lymphoma, unscheduled DNA synthesis, and dominant lethal assays, revealed no evidence for mutagenicity and no evidence for significant genetic risks associated with JP-8 jet fuel.[10]

Acute toxicity testing of JP-8 determined that this jet fuel is non-irritating to the eyes, slightly irritating to the skin and has a weak sensitizing potential.[11] By contrast, Jet A, when tested for acute toxicity, revealed no sensitization after dermal exposure, minimal irritation to eyes and a mild skin irritation potential.[12] A developmental toxicity study of JP-8 indicated that JP-8 is not a teratogen in the rat.[13]

Long term toxicity testing of JP-8 was conducted by exposing Fischer-344 (F-344) rats and C57BL/6 mice to JP-8 vapors at 0, 500, and 1,000 mg/m$^3$ on a continuous basis for 90 days, followed by recovery until approximately 24 months of age. Evaluation of data revealed limited toxicity and no tumor

formation. The toxicity seen after exposure to JP-8 was due to the male rat specific alpha 2-microglobulin protein droplet nephropathy. It is now recognized that the nephropathy seen in male rats is not expected to occur in humans because humans do not produce alpha 2-microglobulin. Therefore, the limited toxicity seen in the JP-8 repeated dose study was not relevant to humans.[14]

Male rats were dosed with neat (undiluted) JP-8 (0, 750, 1500, 3000 mg/kg) daily by oral gavage for 90 days. This study revealed a significant dose-dependent decrease in body weights of rats exposed to JP-8. The male rat-specific alpha 2-microglobulin nephropathy was observed by histopathologic examination. A number of statisically significant changes were also seen in blood and urine that were neither dose-dependent nor biologically significant. Additional treatment-related effects were gastritis and perianal dermatitis. Although there were no histopathological or weight changes in the livers of exposed rats, there was an increase in the liver enzymes aspartamine transferase (AST) and alanine transferase (ALT). The elevated enzymes did not increase with increasing doses of JP-8.[15] Increases in the liver enzymes, AST and ALT, have not been seen in previous subchronic jet fuel vapor studies.[6,14,16,17] Parton *et al.*[18] reported no increase in AST or ALT after exposure of male rats to 500 or 1000 mg/m$^3$ aerosolized JP-8 for one hour daily for either 7 or 28 days. In addition, no liver pathology was reported after exposure to aerosolized JP-8.

Female rats were dosed with neat JP-8 (0, 325, 750, 1500 mg/kg) daily by gavage for a total of 21 weeks (90 days through mating plus gestation and lactation) in an effort to assess adverse effects which may be associated with prolonged exposure to this fuel.[19] Results of this study revealed a significant dose-dependent decrease in body weights of the female rats. Significant organ weight ratio increases were also seen for the liver:body, liver:brain and kidney:brain weights. Although liver enzymes were elevated in the male rat oral study, there was no increase in liver weight.[15] Liver and spleen weights were also not different between control and male and female exposed rats after inhalation exposure to vapors of JP-8 for 90 days.[14] After inhalation of aerosolized JP-8, liver weights were not significantly higher than control rats, but relative liver weights were elevated in the high dose group (1000 mg/m$^3$) in both the 7 and 28 day repeated dose studies and in the low dose group (500 mg/m$^3$) in the 28 day study.[18] There were no changes in urological, hematological or clinical chemistry parameters. Corresponding histopathologic changes and increases in liver enzymes (ALT, AST) were not observed although there was an increase in liver weight. Significant pathological changes were limited to squamous hyperplasia of the stomach and perianal dermatitis.[19]

An attempt was also made to look for biomarkers of chemical exposure. Stress proteins, such as heat shock and glucose-regulated proteins (Hsp and Grp), were thought to be potential biomarkers of chemical exposure. The initial plan was to generate two-dimensional polyacrylamide gel electrophoresis (2-DE)

protein maps of human serum samples from control and exposed individuals and to analyze the resulting patterns for stress protein expression. The classic "stress proteins" are typically intracellular proteins, many are associated with membrane organelles, and do not leak into extracellular fluids such as serum. However, because the human serum protein 2-DE pattern is being characterized, it was still possible to examine the pattern for any detectable shifts or changes in spot size/shape resulting from exposure, paying particular attention to the "Acute-Phase Proteins (APP)." The APP group of >30 proteins serve as serum markers of the acute phase response, a complex set of neurological, endocrine, and metabolic alterations that occur locally and systemically following injury, infection, immunologic reaction, and inflammation. Therefore, the APP were examined as potential biomarkers of exposure to jet fuel.

A number of effects have been seen in animal studies involving jet fuels. One effect, hydrocarbon nephropathy, has not been seen in humans. Skin effects have been variable; no acute animal effects have been reported but incidences have been reported by humans. Liver effects have also been variable between studies or not present. Body weights have been decreased with increasing dose and duration of the study. Neurological effects, although not seen in the animal studies, were possible. Based on the number of potential changes possible after exposure to jet fuels, additional studies with jet fuels are necessary to understand the actual potential hazards for humans.

## II. METHODS

The purpose of the present project was to conduct a pilot study related more to hypothesis generation than hypothesis testing. The study was designed to determine baseline values and then perform a follow-up investigation of the health effects of jet fuel exposure while JP-4 jet fuel was still in use and after conversion to JP-8. The effects to be measured included liver and kidney function, hematopoietic system function, neurocognitive function, and general health.

### Approach

A previous study by the University of Cincinnati at Hill Air Force Base (Hill AFB), Utah, followed a cohort of newly hired men exposed to jet fuel and solvents. Various biomarkers of mutagenic and spermatotoxic effects were measured. This project will be referred to in this paper as the "Biomarkers Study." That effort was funded by the Environmental Protection Agency (EPA), National Institute for Environmental Health Sciences (NIEHS) and the U.S. Air Force.

The current project resulted from a unique opportunity to capitalize on a naturally occurring intervention study. Baseline data on a cohort of exposed and non-exposed subjects was collected in the summer and fall of 1995 while Hill AFB was still using JP-4. Hill AFB then converted to JP-8 late in 1995. The opportunity existed to again sample this same cohort at three points after beginning the use of JP-8. The subjects were sampled according to the schedule in Table 1, with the first samples obtained while JP-4 was still being used and subsequent samples at 3, 6, and 18 months after conversion to JP-8.

**TABLE 1. SCHEDULE OF MEASUREMENT OF EXPOSURE AND EFFECT**

| Exposure[a] | Blood Tests | Physical Exam/Symptoms | MicroCog Test | Industrial Hygiene (exposed) |
|---|---|---|---|---|
| JP-4[b] | X | X | X | X[c] |
| JP-8, 3 months | X | | | |
| JP-8, 6 months | X | X | X | |
| JP-8, 18 months | X | X | X | X |

a=non-exposed subjects were tested on the same schedule, but no industrial sampling was done
b=n=17 for all groups
c=IH was done in previous study (ref. 21) and included 5 of the current subjects

## Subjects

Between 1991 and 1995 the University of Cincinnati conducted extensive monitoring of active duty and civilian personnel exposed to jet fuel as part of the Biomarkers Study. Both full period air sampling and expired breath analysis revealed the presence of measurable levels of benzene and petroleum distillates in these job categories while the Air Force was using JP-4. This study targeted the highest exposed personnel from the previous project including F-16 ground crews (known as Crew Chiefs), aircraft fuel distribution personnel, F-16 fuel system mechanics and F-16 sheet metal workers.

Sample size estimates for the study were calculated using the mean and standard deviation for alkaline phosphatase, for which there were good estimates of variability as it was one of the blood values hypothesized to be affected by fuel exposure. It was determined that 18 subjects who were exposed to jet fuel were required for this study. An additional 18 subjects who were not exposed to jet fuel or other hydrocarbons other than gasoline were recruited from personnel in the Aerospace Medicine Squadron and were matched to the exposed subjects by gender and age within 3 years.

The 5 subjects from the Biomarkers project who were still at Hill AFB and working in exposed jobs were recruited for this project. An additional 13 volunteers were recruited from the same shops and flights to bring the number in the exposed group to 18 and to include women. The additional subjects added to the cohort had to have been working in their respective career field and current assignment for at least 6 months in order to qualify for this study. Seventeen of the eighteen exposed subjects completed all four sampling periods. One subject was transferred to another base after the first sampling period and his results were omitted from the analysis. Five non-exposed subjects were lost to the study due to transfer from the base or leaving the service. Since the power of the study was computed on 18 subjects, the power was reduced and may have been insufficient to detect differences.

Human subjects review and approval for the project was obtained from the Hill Air Force Base Institutional Review Committee, the U. S. Air Force Surgeon General, Office of Clinical Investigations and the University of Cincinnati Institutional Review Board.

## Exposure Measurement

Workplace airborne exposure had been measured on the participants in the Biomarkers study twice while JP-4 was being used. Exposure to JP-8 was measured in the same manner during this study during the week of the fourth blood collection. A personal industrial hygiene air sampling pump was used to collect exposure samples on all exposed subjects. The equipment remained in

place for the full shift through all breaks unless the subject left the area of exposure, in which case it was reattached upon return. The industrial hygiene technician repeatedly observed the workers throughout the sampling period to prevent tampering and to check the operation of the equipment, especially the flow rates of the pumps. Some personnel wore respiratory protection for various lengths of time as required by the Air Force, thus the exposures reported would be higher than the actual inhalation exposure.

Sampling was conducted by a contractor who was a certified industrial hygienist (CIH) using standard National Institute of Occupational Safety and Health (NIOSH) procedures as in the Biomarkers project. The purpose of the sampling was to quantitatively analyze three specific aromatic hydrocarbons and two other groups of organic compounds. The three aromatic hydrocarbons were xylene, toluene, and benzene. The organic compounds were JP-8 jet fuel, analyzed as naphthas, and the polynuclear aromatic hydrocarbons (PAHs) acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)fluoranthene, benzo(e)pyrene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenzo(ah)anthracen, fluoranthene, fluorene, indeno(123-cd)pyrene, naphthalene, phenanthrene, and pyrene. These 16 compounds are included in the NIOSH method for PAH analysis. These three types of analytes (aromatic hydrocarbons, naphthas, and PAHs) were selected because they represented the chemicals or mixtures of interest in raw jet fuel, purging fluid and exhaust. There was interest in the unburned versus burned fuel because of complaints of burning eyes, nose and throat irritation when ground crew were in the hardened aircraft shelters during startup.

Sample pumps were calibrated both before and after their use to ensure air flow rates were accurately known. Sampling equipment was checked periodically throughout the day to ensure it was functioning properly and to determine if the flow rate remained relatively constant. All samples were delivered to Armstrong Laboratory Analytical Services, Brooks Air Force Base for analysis under standard chain-of-custody procedures.

Personal sampling trains consisted of a MSA Flow Lite low flow pump in series with an adjustable Gilian manifold. Each port on the Gilian manifold could be individually set for different flow rates. The number of ports used on each manifold varied depending upon which group of individuals were being sampled. Those individuals exposed to vapors from unburned JP-8 fuel or purging fluid used only one port on their Gilian manifold. This port was connected to a single SKC 226-01 charcoal tube and adjusted for a flow rate of approximately 0.2 liters per minute. This charcoal tube was analyzed for xylene, toluene, benzene, and JP-8 as naphthas. Those individuals exposed to both burned and unburned JP-8 fuel used two ports on their Gilian manifold. One port was connected to a single SKC 226-01 charcoal tube as described above. The other port was connected to a 2.0 micron, 37 millimeter (mm) teflon filter, SKC 225-17-07, assembly in series with a

SKC 226-30-04, XAD-2 tube. The teflon filter cassette was positioned upstream of the XAD-2 tube. The flow rate through this port was approximately 0.6 liters per minute.

In order to determine the source of naphthas, solvents and benzene, bulk samples were obtained from the chemicals (fuel and purging fluid) to which each group of subjects were exposed. The samples were obtained at several points between the fuel delivery truck and the aircraft, including purged and unpurged wing and fuselage tanks. The samples were analysed for the relative composition of the same chemicals we analyzed for in the air samples: naphthas, xylenes, toluene and benzene.

Samples were analyzed on a Hewlett-Packard 5890GC/5971MSD (GC-MSD). A five point calibration curve was made for each BTEX constituent. The r values ranged from 0.922 to 0.979 for the five curves. The samples were run neat (undiluted), the area counts were inserted into the appropriate equation and the quantity of the compound of interest was calculated. An internal standard was not used. The samples and calibration standards were run one after another, without interruption. Chromatographic characterization was then accomplished on the samples, diluted 1/10 in a solvent and run on the GC-MSD.

## Outcome Measurements

### Liver/Kidney Function Tests

Blood collection was performed by standard clinical procedures and required less than 30 ml of whole blood. Air Force regulations regarding blood borne pathogens were enforced as were the requirements set by the IRB and Air Force Surgeon General under the human use protocol. The blood samples were analyzed for liver function including ALT, alkaline phosphatase, and AST and kidney function including blood urea nitrogen (BUN) and creatinine.

### Serum Proteins

Serum samples were solubilized in an SDS-urea solubilization buffer. Sample proteins were separated by two-dimensional gel electrophoresis (2-DE) using the 20 x 25 cm ISO-DALT[7] 2D gel system operating with 20 gels per batch. All first dimension isoelectric focusing (IEF) gels were prepared using the same single standardized batch of ampholytes (BDH Resolyte 3.5-10) selected for ongoing 2-DE serum protein database work. Ten to twenty microliters of solubilized protein sample were applied to each gel and the gels were run for approximately 25,000 volt-hours using a progressively increasing voltage protocol implemented by a programmable high voltage power supply.

A computer-controlled gradient casting system was used to prepare second dimension SDS gradient slab gels in which the top 5% of the gel was 11%T acrylamide, and the lower 95% of the gel varied linearly from 11% to 17%T. Each gel was identified by a computer-printed filter paper label polymerized into the gel. First dimension IEF tube gels were loaded directly onto the slab gels without equilibration. Second dimension slab gels were run in groups of 20 in a DALT slab electrophoresis tank at $10^{N}$C. The runs lasted approximately 18 hr at 160V.

Following SDS electrophoresis, slab gels were stained for protein using a colloidal Coomassie Blue G-250 procedure in covered plastic boxes, with 10 gels per box. This procedure involved fixation in 1.5 liters of 50% ethanol/2% phosphoric acid overnight, three 30 minute washes in 2 liters of cold tap water, and transfer to 1.5 liters of 30% methanol/17% ammonium sulfate/3% phosphoric acid for one hour followed by addition of a gram of powdered Coomassie Blue G-250 stain. Staining required approximately 4 days to reach equilibrium intensity.

Each stained slab gel was digitized at 125 micron resolution, using an Ektron 1412 scanner and high-intensity light box. Raw, digitized gel images were archived on high-density DAT tape and a greyscale videoprint prepared from the raw digital image as hard-copy backup of the gel image. Each 2-DE gel was processed using the Kepler software system with procedure PROC008 which provided a spotlist giving position, shape and density information for each detected spot. This procedure made use of digital filtering, mathematical morphology techniques and digital masking to remove background, and used full two-dimensional least-squares optimization to refine the parameters of a 2D Gaussian shape for each spot. Processing parameters and file locations were stored in a relational database, while various log files detailing operation of the automatic analysis software were archived with the reduced data.

The specific experiment package was constructed using the Kepler experiment definition database to assemble groups of 2-DE patterns corresponding to the various control and experimental groups associated with each experiment. Each pattern was matched to the appropriate "master" pattern, thereby providing future linkage to a human serum protein 2-DE database under development. The groups of gels making up an experiment were then scaled together (to eliminate quantitative differences due to gel loading or staining differences) by a linear procedure based on a selected set of spots. These spots were selected by the GOODSCALE procedure, which selects spots which are Gaussian converged, have a good initial intra-group coefficient of variation (CV), have a good (non-elongated) shape, an integrated density between certain limits (avoiding very small or overloaded spots), and were detected on almost all gels of the set.

Groupwise statistical comparisons (Student's t-test) were made graphically and interactively and the results displayed in montage format using the KPL42 module. Graphical results of individual spot statistics and spot maps were printed in postscript while raw gel images and spot profiles were printed using a 64 level greyscale videoprinter.

**Hematopoietic System Response**

A complete blood count (CBC) with platelet count was performed to assess the effect on the hematopoietic system due to jet fuel exposure.

**General Health Effects**

A focused physical exam specifically examining the eyes, skin, liver and respiratory tract was conducted by a senior Air Force occupational medicine physician or an occupational medicine resident or staff physician under his direction. The skin exam was considered of particular concern due to complaints from personnel with JP-8 exposure. A health history was collected at the time of the physical to assess skin, respiratory and eye problems. Past acute and chronic medical conditions including hepatitis were documented. At the time of the exam, a survey of occupational health problems, a brief work history, social history, and medication use was completed by the subjects to document length and type of exposure, alcohol use, smoking, and medications including over the counter drugs.

**Neurocognitive Function**

The MicroCog neurocognitive functioning test was administered three times, in conjunction with the physical exams. This computer-administered test is user-friendly, and easily completed after a brief orientation.

The results of the MicroCog test are adjusted for age and education level and the instrument was standardized on a sample of 810 subjects at Lackland AFB, Texas where it has been used on Persian Gulf Syndrome patients. It is also being used to test new recruits at Lackland AFB, Texas and on aircraft pilots at Kelly AFB, Texas. A study using MicroCog on about 4,000 Airman Basic recruits was conducted for a DoD study of gender differences.

Five neuropsychological areas are examined by the test: (1) attention/mental control; (2) memory; (3) reasoning/calculation; 4) spatial processing; and (5) reaction time. Scoring of the exam was done from a computer file generated at the time of testing.

## III. RESULTS AND DISCUSSION

The characteristics of the exposed and non-exposed subjects are given in Table 2. There were no statistically significant differences between the groups in percent who smoked or consumed alcohol by Fisher's Exact t or the Chi-Square test.

**TABLE 2. DEMOGRAPHIC CHARACTERISTICS OF SUBJECTS**

| DEMOGRAPHICS | EXPOSED | NON-EXPOSED |
|---|---|---|
| Mean Age | 32.8 (SD[a]=10.3) | 32.3 (SD=9.4) |
| Number of Females | 5 | 5 |
| Number of Males | 12 | 13 |
| Percent Smokers | 29% | 22% |
| Percent Caucasian | 94% | 94% |
| Alcohol Use (in percent): | | |
| None | 47% | 39% |
| Less than 1 drink/week | 29% | 6% |
| 1 or more drinks/week | 24% | 56% |

a=SD=standard deviation

### Industrial Hygiene Sampling

Industrial hygiene personal air sampling results are displayed in Table 3 as vapors, aerosols and particulates while JP-8 was in use. The subjects were grouped by 5 categories of jobs based on expected type and amount of exposure. Crew Chiefs (Group 1) work on the flight line where they launch, recover and service F-16 jet fighters. Petroleum Distribution personnel (Group 2) fill large tank trucks with JP-8 and drive the trucks to the flight line to fuel aircraft. The 388th Fighter Wing Fuel Shop (Group 3) is responsible for repair of both integral (within the fuselage and wings of the aircraft) and external (removable wing tanks) fuel tanks and associated fuel system components on the F-16 while the aircraft is in flying status. When work is required on the integral tanks, they are emptied and purged with air. The external tanks are purged with PD-680, which is a petroleum distillate. Use of the solvent PD-680 also poses a potential exposure to hydrocarbons, some of which may be identical to the hydrocarbons in jet fuel. This solvent could contribute to the naptha analysis. In a purged F-16 there are places where purging fluid never reaches. Some purging fluid is always left behind and must be wiped up with towels. The civilian F-16 fuel workers (Group 4) perform depot level maintenance on purged aircraft only, replacing or refurbishing only the integral fuel systems. The Civilian Shop only uses purging fluid once and then recycles it to get rid of the volatile hydrocarbons. F-16 sheet metal workers (Group 5) perform depot level sheet metal replacement and repair. This includes working around

open integral fuel cells which have been purged. Sheet metal workers may also be exposed to a variety of other chemicals and solvents such as toluene and xylenes in paints. For all of the categories of workers in this study there is a potential for exposure to more than just jet fuel. The individual sampling results are given in Appendix A.

There are striking similarities with data obtained in the Biomarkers project while JP-4 was in use [21] (Table 4). Vapor levels of naphthas in the breathing zone air are extremely low in both studies and a small fraction of the standard (300 ppm naphthas for NIOSH and Occupational Safety and Health Act (OSHA)). Fuel exposure is highest in the fuels system repair and sheet metal workers in both studies, rather than the flight line crew members or fuel delivery personnel, as was predicted since they work outside. These three groups are reported separately in this JP-8 study, but fuel delivery and fuel repair were grouped together in the Biomarkers Project when JP-4 was being used. The F-16 civilian fuel system repair subjects had the highest measured naphtha levels in the current study. The military fuel repair shop and the F-16 civilian sheetmetal shop had comparable levels of naphtha exposure, which was not expected, since the civilian shop works on aircraft that have been purged for some time. The source of the naphthas is threefold: there is fuel contaminating the purge fluid, the purge fluid, and puddles of fuel in the aircraft. Both the purge fluid and jet fuel were analyzed and reported as naphthas.

No benzene was measured via personal air sampling while exposed to JP-8, in contrast to the levels under JP-4 exposure, which were 0.05 ppm on the average. Aerosols and particulates were also measured on subjects working on the flightline where such exposure would be expected. No particulates were detected. For the aerosols only acenaphthene and naphthalene were above the limit of detection. However, it is very difficult to sample for particulates and aerosols with just personal air sampling methods. The fact that no particulates were detected does not necessarily mean that they were not present. The same applies to aerosols; detection of only two aerosols does not mean that others may not have been present.

Naphthalene has a permissible exposure limit (PEL) of 10 ppm (52 $mg/m^3$) and is classified as A4 - not classifiable as a Human Carcinogen, inadequate data. It is an experimental teratogen with experimental reproductive effects. It is an eye and skin irritant, can cause nausea, headache, diaphoresis, hematuria, fever, anemia, liver damage, vomiting, convulsions, and coma. Poisoning may occur by ingestion of large doses, inhalation, or skin absorption . [22]

Acenaphthene is a PAH often found in jet fuel sampling and jet fuel contaminated soil. It is moderately toxic by the intraperitoneal route and mutation data have been reported.

The upper respiratory, skin and eye irritation symptoms reported for naphthalene and acenaphthene are similar to symptoms reported by subjects in this study and by workers at other bases when in contact with jet fuel (Personal Communication, Dr. Gould). This study only sampled for aerosols on the flight line workers, because that is where clouds of aerosol have been seen during engine starts at cold temperatures, typically below zero. The start of a single engine under cold conditions may completely engulf the aircraft in aerosol. Since the sampling media were expensive no sampling was conducted for aerosols on the indoor workers. Unfortunately, indoor workers exhibited some of the same symptoms reported by the flight line personnel. Possibly inhalation of vapors or skin contact produces the effect and the compounds do not necessarily need to be aerosolized.
The results of the bulk exposure source analysis and chromatographic characterization are presented in Table 5. JP-8 jet fuel at Hill AFB does contains benzene at a level of 0.01% (104 to 142 ppm). The content of toluene ranges from 0.03% in the liquid remaining in purged tanks to 1.3% in the unopened wing tank. Xylenes were present at levels from 1.0% in purged fuel tank to 8% in the unopened wing tank. Ethylbenzene is also present at levels between 0.03% and 0.09%. Ethylbenzene is an irritant to eyes, skin, and mucous membranes and can affect the central nervous system. These volatile aromatic hydrocarbons persisted even in liquid remaining in purged fuel cells which had been open for 6 hours.

The tank of PD-680 purging fluid from the 388th Fuels Shop had the chromatographic picture of JP-8 mixed with purging fluid, because it had been used previously and normally is recycled until the flash point exceeds the lower explosive limit (L.E.L.). The fuel bowser (sample 3) is a small piece of equipment which vacuums fuel out of the bottom of the integral fuel cells on the F-16 in the 388$^{th}$ Fuels Shop.

## Hematopoietic System

All parameters of the clinical blood analysis were tested for normality using the Shapiro-Wilks Test and homogeneity of variance by the Levine's Test. All tests were non-significant, indicating the variables were normally distributed and variances were equal, except for basophils, which showed non-homogeneous variance. Analysis of both exposure group and time effect was by a time series covariance model in which the correlations of the repeated measurements decline as a function of unequally spaced sampling intervals. Although the main effect of smoking was not significant for any of the variables, we controlled for smoking status because it is such an important confounder. Interaction terms between the main effects were not included because they were not found to be significant. With this analysis, red blood cell values of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly different ($p<0.0001$) between exposed and non-exposed before and after Bonferroni corrections (p x 18). Values both before and after correction are cited because not all statisticians ascribe to the use of this

correction, and because this is a pilot study, hopefully pointing the way for future investigators. The mean, standard deviation and sample sizes for the 3 significant parameters are given in Table 6 and Figures 1, 2 and 3. The results for all parameters are shown in Appendix B.

The significant blood parameters are related to each other in the following manner:

$$MCV = \frac{\text{volume of packed red cells}}{\text{red cell count}}$$

$$MCH = \frac{\text{hemoglobin}}{\text{red cell count}}$$

$$MCHC = \frac{\text{hemoglobin}}{\text{volume of packed red cells}}$$

TABLE 3. INDUSTRIAL HYGIENE AIR SAMPLING RESULTS DURING JP-8 USE GIVEN AS MEAN AND (STANDARD DEVIATION) IN PPM (n = number of industrial hygiene samples)

| | Vapors (n=33) | | | Aerosols (n=11) | |
|---|---|---|---|---|---|
| **Job Group** | **Naphthas** | **Toluene** | **Xylenes** | **Acenaphthene** | **Naphthalene** |
| All Exposed Subjects n=33* | 1.33 (1.75) | 0.11 (0.29) | 0.14 (0.61) | not measured | not measured |
| Crew Chiefs & Weapons n=9** | 0.13 (0.11) | 0.01 (0.003) | 0.02 (0.005) | 0.000032 (0.0001) | 0.0006 (0.0004) |
| Fuel Delivery n=2** | 0.09 (0.05) | 0.01 (0.003) | 0.02 (0.002) | 0.0002 (0.0002) | 0.0007 (0.0009) |
| 388th FW Fuels Shop n=5 | 1.28 (1.08) | 0.04 (0.02) | 0.05 (0.03) | not measured | not measured |
| F-16 Fuels Repair (Civilian) n=11 | 2.62 (2.33) | 0.13 (0.16) | 0.05 (0.05) | not measured | not measured |
| F-16 Sheet Metal (Civilian) n=6 | 1.16 (1.0) | 0.31 (0.62) | 0.62 (1.44) | not measured | not measured |

* Benzene was also measured on all subjects, but was below the limit of detection in all cases.

** Particulates were also measured, but were also below the limit of detection.

TABLE 4. INDUSTRIAL HYGIENE SAMPLING RESULTS DURING JP-4 USE (BIOMARKERS STUDY*) GIVEN AS MEAN, (STANDARD DEVIATION) AND SAMPLE SIZE (n = number of subjects, ns = number of IH samples)

| Job Group | Fuel (ppm) | Total Solvents (ppm)** | Benzene (ppm) |
|---|---|---|---|
| All Exposed Subjects | 3.2 (12.7) | 1.6 (6.9) | 0.05 (0.20) |
| n=50 | ns=182 | ns=286 | ns=176 |
| Painters | 1.4 (2.6) | 2.4 (4.3) | 0.0 (0.0) |
| n=6 | ns=18 | ns=35 | ns=18 |
| Flight Line | 1.5 (2.7) | 0.46 (1.4) | 0.02 (0.04) |
| n=23 | ns=60 | ns=126 | ns=60 |
| Jet Fueling*** | 5.2 (20.1) | 1.2 (3.7) | 0.08 (0.27) |
| n=15 | ns=68 | ns=89 | ns=62 |
| F-16 Sheet Metal | 3.3 (5.9) | 5.9 (17.5) | 0.05 (0.27) |
| n=6 | ns=36 | ns=36 | ns=36 |

* Reference 21

** Total Solvents includes xylenes, toluene, methyl ethyl ketone, methylene chloride and 1,1,1-trichloroethane

*** Includes petroleum distribution and both civilian and military fuel system repair

TABLE 5. ANALYSIS AND CHARACTERIZATION OF BULK SAMPLES FROM EXPOSURE SOURCES IN PPM

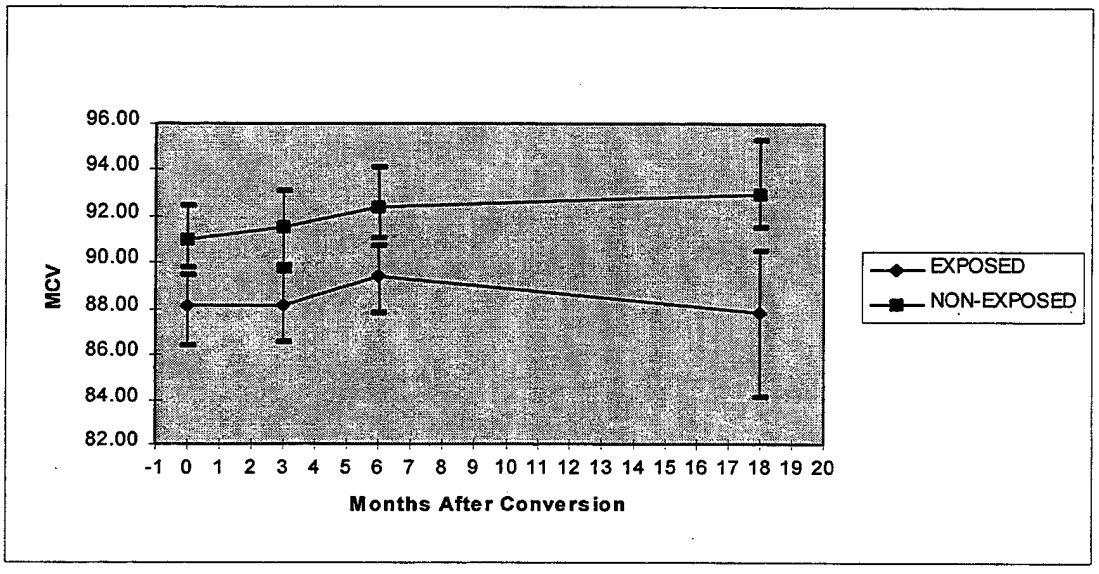
| Sample | Origin of Sample | Characterization | Benzene | Toluene | Ethylbenzene | Xylenes |
|---|---|---|---|---|---|---|
| 1 | fuel delivery truck | JP-8 | 118 | 766 | 730 | 4614 |
| 2 | F-16 integral fuel cell open 4 days | JP-8 | 118 | 1049 | 867 | 6600 |
| 3 | fuel bowser in Fuel Shop | JP-8 | 111 | 750 | 691 | 4168 |
| 4 | tank of purging fluid (PD 680) | Mixed purging fluid and JP-8* | 104 | 545 | 504 | 2990 |
| 5 | unopened F-16 wing tank | JP-8 | 142 | 1302 | 970 | 8707 |
| 6 | purged F-16 fuel cell open 4 weeks | purging and possibly hydraulic fluid | ND | ND | ND | ND |
| 7 | purged F-16 fuel cell open 6 hours | JP-8, purging and hydraulic fluid | ND | 318 | 315 | 1047 |
| 8 | purged C-130 fuel cell | Mixed purging fluid and JP-8 | ND | 420 | 387 | 1788 |

* purging fluid is used many times until the flashpoint reaches the lower explosive limit due to mixing with fuel.

TABLE 6. MEANS (STANDARD DEVIATIONS , SAMPLE SIZES) OF SIGNIFICANT BLOOD PARAMETERS BY GROUP AND SAMPLING PERIOD

| Blood Parameter | Sampling Period | Exposed Group mean (SD, n) | Non-Exposed Group mean (SD, n) |
|---|---|---|---|
| **MCV** | JP-4 | 88.1, (3.4, 17) | 91.0, (3.0, 18) |
| mean corpuscular vol. | JP-8, 3 months | 88.1, (2.9, 17) | 91.5, (3.3, 18) |
| | JP-8, 6 months | 89.4, (3.2, 17) | 92.4, (3.4, 18) |
| | JP-8, 18 months | 87.8, (7.2, 17) | 92.9, (4.0, 13) |
| **MCH** | JP-4 | 30.2, (1.2, 17) | 31.1, (0.9, 18) |
| mean corpuscular | JP-8, 3 months | 31.0, (1.1, 17) | 32.0, (1.3, 18) |
| hemoglobin | JP-8, 6 months | 31.3, (1.2, 17) | 32.0, (1.2, 18) |
| | JP-8, 18 months | 29.7, (1.6, 17) | 30.3, (1.2, 13) |
| **MCHC** | JP-4 | 34.2, (0.7, 17) | 34.2, (0.7, 18) |
| mean corpuscular | JP-8, 3 months | 35.3, (0.7, 17) | 35.0, (0.8, 18) |
| hemoglobin conc. | JP-8, 6 months | 35.0, (0.5, 17 | 34.6, (1.0, 18) |
| | JP-8, 18 months | 33.4, (0.7, 17) | 32.6, (0.7, 13) |

Figure 1. Mean Corpuscular Volume (MCV) of Exposed and Non-Exposed Subjects Before and After Conversion to JP-8 with 95% Confidence Intervals



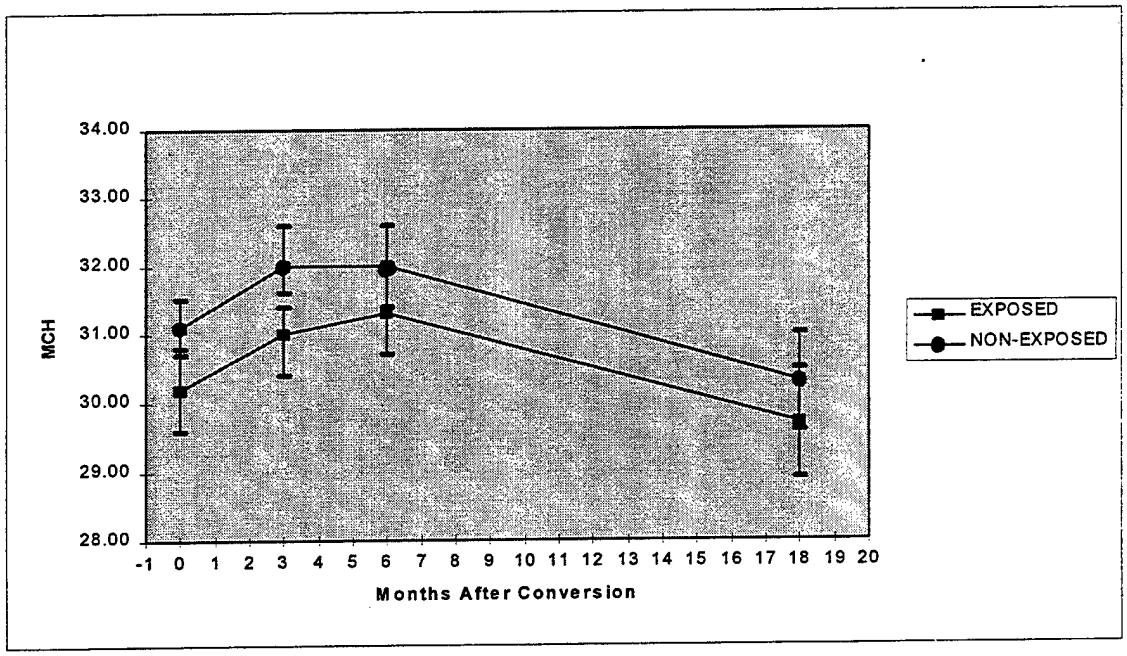



Figure 2. Mean Corpuscular Hemoglobin (MCH) of Exposed and Non-Exposed Subjects Before and After Conversion to JP-8 with 95% Confidence Levels

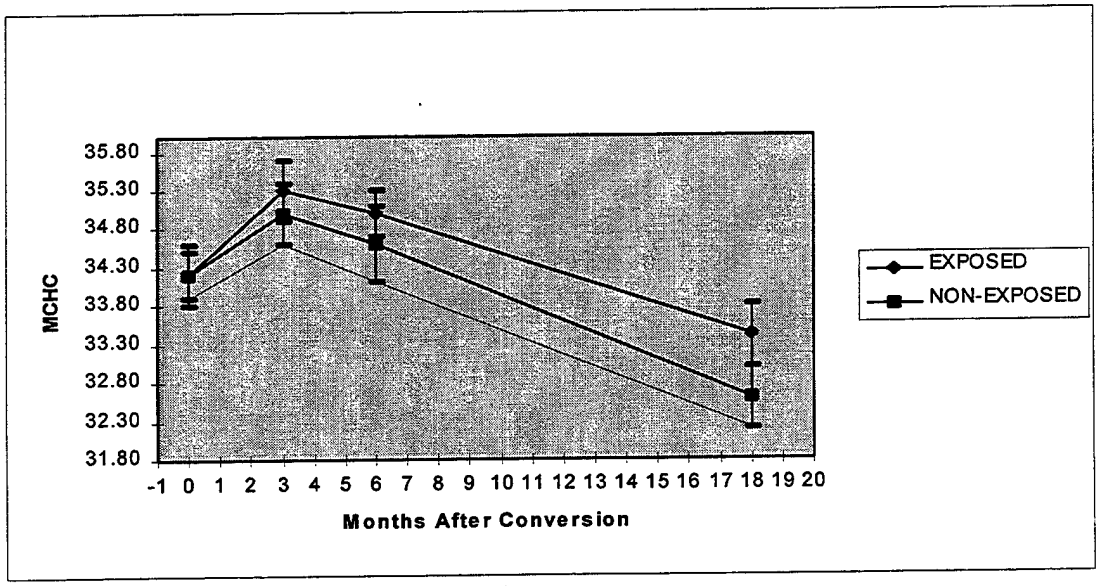



Figure 3. Mean Corpuscular Hemoglobin Concentration (MCHC) of Exposed and Non- Exposed Subjects Before and After Conversion to JP-8 with 95% Confidence Limits

The means for MCV and MCH are consistently higher in the non-exposed group across the four time periods. The reverse is true for MCHC after sample 1(JP-4). MCH and MCHC show an increase at the second sampling period, even in the controls, who were sampled at the same times, probably indicating either seasonal variation or a change in the laboratory equipment or procedures. The decreases over time are significant for MCH and MCHC when each post-JP-8 period was tested against the JP-4 baseline by analysis of variance ($p \leq 0.0001$ without Bonferroni correction). The change (increase) in MCV was only significant for sample 3 versus sample 1. These changes also may be due to seasonal variation, weather, pollution, or other factors operating on both the exposed and non-exposed populations.

Although no other studies of jet fuel effects on hematology could be found, there are studies on the effects of benzene and solvents such as PD-680 (Stoddard Solvent). Benzene is of particular concern because it is known to localize in the bone marrow, blocking proliferation of cells and is also known to have mutagenic effects which may result in aplastic anemia with chronic exposure.[23] Hematocrit, total leukocyte count and platelet count may be decreased by benzene exposure. The anemia is usually normocytic and normochromic, although macrocytosis may be found. Certain leukemias may also be caused by benzene exposure. [24]

Studies of 49 female workers in shoe manufacturing exposed to benzene and toluene reported increased MCV and decreased MCHC compared to 27 controls. [25] An earlier study of the same population [26] showed the same results, with the exception of no effect when exposure to benzene was < 5 ppm. A study of 221 styrene-exposed workers showed the same changes when compared with 104 controls. [27] Moszcynski and Lisiewicz [28] reported that 106 workers exposed to a combination of benzene, toluene and xylene for up to 122 months had decreased MCH and MCHC. In addition, leukocytes were decreased, particularly T-, non-T, and non-B cells. Mice fed crude and other petroleum oils showed an increase in MCHC. [29]

Investigations of the effect of confounding factors such as smoking, alcohol consumption, exercise, temperature and medications do little to explain the results. Rosello *et al.* [30] reported smoking increased MCV and MCH and had no effect on MCHC in 507 healthy subjects. Heidemann [31] compared 120 chronic alcoholics with the normal population and showed an increase in MCH. Exposure to both organic solvents and alcohol had no effect on MCV in a study by Moen *el al.* [32] Measurements on 4317 males 20 to 64 years of age in Israel showed that in August both MCV and hematocrit were decreased compared to the cooler months of the year. [33] Cold weather resulted in an increase in MCHC, with MCV remaining unchanged in a study of 9 Navy men undergoing extended arctic weather training in Utah and Alaska and 27 men in a cold air acclimatization program. [34] Exercise showed different effects in two studies. Kondo *et al.* [35] reported an increase in MCHC in 42 female college students enrolled in a 13 week exercise program. The

opposite was reported by Radomski *et al.* [36] on soldiers who marched 35 km per day for 6 days. A decrease in hemoglobin, MCH, MCV, and MCHC was called "sports anemia."

It must be stressed here that the majority of the values of MCV and MCH measured in this study were within normal clinical limits. The exposed group had one person with low MCV and low MCH in period 4. This subject was evaluated for undiagnosed internal bleeding. There were 5 non-exposed subjects with high MCH in periods 2 and 3. Two of these were heavy drinkers, 1 was a body builder, and 1 was being followed for low RBC and hematocrit. His iron and total iron binding capacity were within normal limits.

In order to explain the data better and to find out why MCV was different for exposed and non-exposed workers in Figure 1, we attempted to locate blood results done before, or near the time, when the subjects entered fuel-exposed jobs. Charts were not available for 15 of the subjects (9 non-exposed and 6 exposed). When charts were available, only 10 of the exposed had a previous CBC near the beginning of their exposure, and one of these was the person with suspected internal bleeding. That data was excluded from the analysis. Only 4 of the non-exposed had blood work and one of those had previously worked in aircraft repair, and that data was excluded. The amount of change in MCV and MCH from pre-exposure lab values to the fourth sample was averaged for the 2 groups. The exposed had an average increase in MCV of 1.5 and a decrease in MCH of 0.14. The non-exposed had an average increase in MCH of 3.5 and increase in MCH of 1.5. No statistical tests were run on these numbers due to the very small sample sizes (9 and 3).
This superficial analysis supports the impression that there is a divergence over time in these blood measures for exposed and non-exposed subjects.

The values for indicators of liver function, alanine transferase (ALT) and alkaline phosphatase, are shown in Appendix B. There is no significant difference between exposed and non-exposed subjects, nor between smokers and non-smokers in the repeated measures analysis of variance.

## Serum Proteins

Approximately 600 of the most prominently expressed proteins are observed in the 2-DE system. No exposure related alterations were detectable by the 2-DE results in the analysis of the serum samples. No new potential biomarker was discovered in this study. In addition, frozen whole blood samples were shipped from Hill AFB but pure erythrocyte fractions were unattainable due to extensive cell lysis and mixing with serum proteins. Therefore 2-DE analysis of erythrocyte patterns was not possible. The 2-DE patterns simply resembled alternative versions of the serum patterns already obtained. Cellular components of the blood may yield more significant indicators of exposure-related toxicity if they are isolated from the serum

and solubilized as a homogenous cell fraction. Sampling could be conducted with a revised protocol for sample preparation.

These data clearly demonstrate the heterogeneity of the human serum samples studied. Given the sample variation, it is not surprising that neither paired nor unpaired t-tests of the protein abundance (integrated spot density) showed any significant differences related to jet fuel exposure. Despite the variation, the resolution and spot detection observed were satisfactory.

The data presented here suggest that any alterations in serum protein patterns associated with conversion from JP4 to JP8 and chronic JP-8 exposure are undetectable by the 2-DE methods used in this study. The full report is attached in Appendix C.

Serum and tissue samples were analyzed from Swiss-Webster mice exposed to aerolsolized JP-8 jet fuel in studies conducted by at the University of Arizona. Preliminary results indicate not only significant tissue effects, but changes in the 2-DE pattern of serum protein expression as a result of JP-8 aerosol inhalation at high concentration. A homogeneous population of exposed animals may be a better initial indicator of the hazards of JP-8 exposure than a group of variant humans or the concentration of jet fuel exposure was not high enough to result in observable effects in humans or the exposures to mice by the University of Arizona group were significantly greater than could be anticipated in field exposure scenarios .

**General Health Effects**

Subjects were asked to report frequency of selected symptoms related to jet fuel exposure during the previous month in conjunction with the physical exams at three times: baseline (JP-4), 6, and 18 months after beginning the use of JP-8. The comparison of the exposed and non-exposed frequency of symptoms is given in Table 7. Frequency responses were tested for changes over the sampling periods using the Cochran-Mantel-Haenszel method, which is appropriate for repeated measures which are ordinally scaled. There were no significant trends (either increasing or decreasing) in the symptom frequency, although the response at some sampling periods differed from others. It is interesting that both the mean and median frequency of most of the symptoms was generally less than once or twice a month for both the exposed and non-exposed groups.

The physical exams failed to show any differences between the two groups of subjects at any of the sampling periods, with one exception. Two of the exposed subjects reported severe rashes and swelling of their knuckles upon exposure to JP-8. One subject was an active duty F-16 Crew Chief and the other was a civilian working in F-16 fuel cell repair. Both of these subjects had been volunteers in the

Biomarkers project and did not have the rash when using JP-4. Barrier cream and nitrile gloves did not protect the subjects against the rash caused by JP-8. Special gloves were obtained from Gore (Dermapor) which are worn under the nitrile gloves and can be washed. This eliminated the problem for the fuels worker. The other subject transferred to a non-exposed job and subsequently left the service.

Dry, itchy skin or rashes were reported a total of 31 times by exposed subjects. The subjects attributed the symptom to jet fuel 5 out of 9 times (56%) while JP-4 was used and 9 out of 21 times (43%) during JP-8 use. The non-exposed subjects reported the same symptoms 20 times. The dry, itchy skin may be due in large part to the extremely dry climate in Utah.

## Neurocognitive Function

The results of the MicroCog test are presented in Tables 7 and 8. Table 8 presents descriptive statistics for the MigroCog indices for both exposed and non-exposed groups. Index scores were corrected for age and level of education of both groups. Group means of the various indices were consistently higher for the non-exposed group as compared to the exposed group means, although not statistically different ($p>0.05$ on all t-tests). Two statistical approaches were taken in examining differences between groups as a function of testing. First, an analysis of covariance was conducted using MicroCog index scores for the third testing as the dependent variable, group (exposed vs. non-exposed) as the independent variable and MicroCog index scores for the first testing as the covariate. These results indicated there were no significant differences between the means of the groups at the third testing ($p>0.05$). In order to investigate possible group differences between the first and second tests, an analysis of covariance was performed using the index scores for the second testing as the dependent variable, group (exposed vs. non-exposed) as the independent variable and the MicroCog index scores for the first testing as the covariate. In these analyses, a statistical difference ($p\leq0.05$) was obtained between the groups for the Reaction Time Index, with the non-exposed group (mean=112.1) scoring higher than the exposed group (mean=105.3). A higher score indicates a faster response.

Secondly, a repeated measures analysis of variance was performed using the MicroCog index scores to investigate if there were group differences from the first to the third testing. Similar to the analysis of covariance results, group mean differences were not statistically significant ($p>0.05$). All the MicroCog indices were statistically significant ($p<0.05$) for times (i.e., the change from first to third testing), with the mean for the third testing always being higher than the first testing.

Examination of the means at the three testing periods for the two groups shows that both groups gained points at retest, suggesting a small practice effect of about 4-5 points. Importantly, there were no significant interaction effects of group by time. These results suggest that the exposed group was not performing at a different cognitive level as compared to the control group (i.e., they were equivalent) and no decrements were found over the test-retest interval. Rather, performance on four of the indices improved for the groups, suggesting a mild practice effect for these measures.

Overall, these results suggest that the exposed individuals were not significantly different from non-exposed individuals after adjusting for baseline scores (the first testing). However, there was a tendency for the exposed group to score lower than the non-exposed group at each of the three test periods. This could be explained by either chance variation or by the fact that the exposed group had all been working with jet fuel for at least six months. Another explanation for the higher group means in the non-exposed group is that the average education level was 14.8 years as opposed to 12.6 years for the exposed group. However, since years of education is related to the cognitive test scores, all scores have been adjusted for level of education. It may be that small or more subtle differences do indeed exist in the cognitive measures of the two groups, but a larger sample size would be required to obtain statistically reliable differences.

TABLE 7. MEAN FREQUENCY OF SYMPTOMS DURING THE MONTH PREVIOUS TO SAMPLING, ON A SCALE OF 0 TO 5*

| | JP-4 | | JP-8, 6 Months | | JP-8, 18 Months | |
|---|---|---|---|---|---|---|
| | Exposed | Non-Exposed | Exposed | Non-Exposed | Exposed | Non-Exposed |
| Skin Rashes | 0.3 | 0.3 | 0.7 | 0.1 | 0.3 | 0.5 |
| Dry, Itchy Skin | 1.7 | 1.6 | 1.8 | 1.5 | 1.7 | 1.7 |
| Eye Irritation | 1.2 | 0.6 | 1.5 | 1.6 | 1.7 | 1.0 |
| Nose Irritation | 1.6 | 0.8 | 1.8 | 1.6 | 1.8 | 1.0 |
| Throat Irritation | 1.0 | 0.6 | 1.4 | 1.2 | 1.3 | 1.0 |
| Cough | 1.0 | 0.8 | 1.2 | 1.7 | 1.6 | 0.3 |
| Shortness of Breath | 1.1 | 0.7 | 0.8 | 1.3 | 1.4 | 0.0 |
| Difficulties Concentrating | 1.3 | 0.9 | 1.6 | 1.2 | 1.4 | 1.2 |
| Headache | 1.0 | 0.5 | 1.6 | 0.9 | 1.2 | 0.8 |
| Dizziness | 1.6 | 1.3 | 1.3 | 1.9 | 1.2 | 1.4 |
| Fatigue | 0.9 | 0.5 | 0.7 | 1.3 | 1.2 | 1.1 |
| Nausea/Vomiting | 0.4 | 0.2 | 0.8 | 0.5 | 0.3 | 0.4 |
| Numbness/tingling hands or feet | 1.2 | 1.2 | 1.4 | 1.5 | 1.4 | 1.6 |

*Frequency of symptoms scale:
0=never happens
1=once or twice per month
2=3 or 4 times per month
3=5 to 9 times per month
4=10 or more times per month
5=every day

TABLE 8. RESULTS OF MICROCOG TESTING UNDER JP-4 AND JP-8

| | JP-4 | | JP-8, 6 MONTHS | | JP-8, 18 MONTHS | |
|---|---|---|---|---|---|---|
| **NON-EXPOSED SUBJECTS** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| | (n=18) | | (n=18) | | (n=13) | |
| Attention/Mental Control | 100.4 | 14.7 | 100.6 | 9.8 | 106.1 | 13.1 |
| Reasoning/Calculation | 97.3 | 11.5 | 98.0 | 13.2 | 104.4 | 17.3 |
| Memory | 104.9 | 14.6 | 105.6 | 10.6 | 111.8 | 10.9 |
| Spatial Processing | 103.4 | 11.9 | 106.4 | 8.5 | 105.6 | 10.8 |
| Reaction Time * | 106.3 | 9.7 | 112.1 | 6.4 | 108.2 | 6.7 |
| Information Processing Speed | 98.5 | 15.3 | 103.3 | 12.1 | 103.8 | 14.8 |
| Information Processing Accuracy | 97.7 | 12.5 | 96.3 | 15.9 | 105.8 | 8.7 |
| General Cognitive Functioning | 97.8 | 13.1 | 99.7 | 9.9 | 106.4 | 12.1 |
| General Cognitive Proficiency | 97.1 | 10.8 | 98.0 | 8.2 | 103.3 | 12.8 |
| **EXPOSED SUBJECTS** | | | | | | |
| | (n=17) | | (n=17) | | (n=16**) | |
| Attention/Mental Control | 94.7 | 21.0 | 96.7 | 17.0 | 103.0 | 13.6 |
| Reasoning/Calculation | 95.4 | 14.7 | 98.4 | 13.5 | 102.5 | 15.6 |
| Memory | 100.9 | 16.7 | 104.1 | 12.1 | 111.3 | 12.2 |
| Spatial Processing | 100.9 | 14.8 | 105.5 | 7.4 | 105.3 | 11.9 |
| Reaction Time * | 105.5 | 10.1 | 105.3 | 12.2 | 107.2 | 9.5 |
| Information Processing Speed | 94.8 | 11.5 | 100.0 | 8.5 | 99.4 | 15.3 |
| Information Processing Accuracy | 95.9 | 18.2 | 97.7 | 14.7 | 107.9 | 14.7 |
| General Cognitive Functioning | 94.2 | 16.9 | 98.6 | 12.1 | 104.9 | 14.6 |
| General Cognitive Proficiency | 90.1 | 12.4 | 94.3 | 10.5 | 99.4 | 14.4 |

* Significant difference between Non-Exposed and Exposed subjects at 6 months of JP-8 with the JP-4 value as the covariate.

** One subject did not take the MicroCog test.

## SUMMARY

The results of this pilot study are not conclusive, but merely suggest the need for further investigation into the long term effects of jet fuel exposure. Exposure to the vapors of JP-8 in the job groups studied at Hill AFB was extremely low (<3% of the naphtha standard) and there was no detectable benzene exposure. After 18 months of JP-8 use, the exposed workers had lower MCH and MCV, and higher MCHC than the non-exposed subjects. Most values were within normal clinical limits. These differences are significant regardless of smoking status, and the subjects have been matched for gender and age. We should consider investigating this trend more thoroughly.

The reaction time index of the MicroCog test was significantly higher (faster response) in the non-exposed group on the second MicroCog test during the third sampling period (6 months after JP-8) with the JP-4 value as the covariate. In addition, the neurocognitive function test, the MicroCog, shows that there is a persistent, although not statistically significant, difference between the groups with the exposed scoring lower than the non-exposed subjects at the baseline and through the last testing period.

Further research is needed to clarify the effects of low level, long term exposure to complex hydrocarbon mixtures such as jet fuel. Medical surveillance of workers is a major concern of the Air Force but this study did not yield a definitive answer to the question of what should be included in such surveillance on fuel-exposed workers. We would recommend an expert panel determine if further action is necessary and what tests would yield reliable information.

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Appendix A: Industrial Hygiene Sampling Results by Individual and Job Group (ppm).

| Group | Subject | Benzene | Naphthas | Toluene | Xylenes | Acenaphthene | Naphthalene |
|---|---|---|---|---|---|---|---|
| 1 | E01 | 0 | 0.000 | 0.009 | 0.022 | 0.00032 | 0.00114 |
| 1 | E01 | 0 | 0.000 | 0.007 | 0.017 | 0.00000 | 0.00057 |
| 1 | E01 | 0 | 0.187 | 0.009 | 0.016 | 0.00000 | 0.00057 |
| 1 | E04 | 0 | 0.310 | 0.014 | 0.029 | 0.00000 | 0.00095 |
| 1 | E04 | 0 | 0.198 | 0.012 | 0.026 | 0.00000 | 0.00000 |
| 1 | E06 | 0 | 0.194 | 0.010 | 0.026 | 0.00000 | 0.00057 |
| 1 | E06 | 0 | 0.123 | 0.008 | 0.018 | 0.00000 | 0.00057 |
| 1 | E16 | 0 | 0.178 | 0.010 | 0.024 | 0.00000 | 0.00095 |
| 1 | E16 | 0 | 0.000 | 0.010 | 0.024 | 0.00000 | 0.00000 |
| 2 | E10 | 0 | 0.196 | 0.012 | 0.023 | 0.00032 | 0.00133 |
| 2 | E10 | 0 | 0.128 | 0.009 | 0.019 | 0.00000 | 0.00000 |
| 3 | E12 | 0 | 1.045 | 0.045 | 0.057 | | |
| 3 | E12 | 0 | 3.034 | 0.031 | 0.055 | | |
| 3 | E13 | 0 | 0.086 | 0.003 | 0.008 | | |
| 3 | E13 | 0 | 1.300 | 0.055 | 0.089 | | |
| 3 | E13 | 0 | 0.959 | 0.046 | 0.058 | | |
| 4 | E02 | 0 | 0.460 | 0.042 | 0.026 | | |
| 4 | E02 | 0 | 1.510 | 0.427 | 0.044 | | |
| 4 | E09 | 0 | 0.583 | 0.045 | 0.030 | | |
| 4 | E09 | 0 | 3.564 | 0.040 | 0.022 | | |
| 4 | E11 | 0 | 1.492 | 0.039 | 0.026 | | |
| 4 | E11 | 0 | 7.102 | 0.482 | 0.047 | | |
| 4 | E14 | 0 | 0.680 | 0.039 | 0.048 | | |
| 4 | E14 | 0 | 2.475 | 0.052 | 0.074 | | |
| 4 | E15 | 0 | 5.892 | 0.167 | 0.190 | | |
| 4 | E15 | 0 | 0.640 | 0.036 | 0.039 | | |
| 4 | E15 | 0 | 4.475 | 0.059 | 0.037 | | |
| 5 | E07 | 0 | 0.339 | 0.027 | 0.028 | | |
| 5 | E07 | 0 | 2.983 | 1.589 | 0.031 | | |
| 5 | E17 | 0 | 0.350 | 0.021 | 0.024 | | |
| 5 | E17 | 0 | 0.983 | 0.150 | 3.565 | | |
| 5 | E18 | 0 | 1.538 | 0.061 | 0.030 | | |
| 5 | E18 | 0 | 0.768 | 0.055 | 0.037 | | |

* Group Codes:
1=Crew Chiefs
2=Petroleum Distribution
3=388th FW Fuels Shop
4=F-16 Fuels (Civilian)
5=F-16 Sheet Metal (Civilian)

Appendix B: Results of CBC with Differential, Liver and Kidney Function

| Parameter | Period | # Samples | Mean | S.D. | Minimum | Maximum |
|---|---|---|---|---|---|---|
| **White Blood Cell Count ($10^3$)** | | | | | | |
| Control | 1 | 18 | 6.767 | 1.680 | 3.3 | 10.2 |
| | 2 | 19 | 6.342 | 1.759 | 3.6 | 10.1 |
| | 3 | 19 | 6.421 | 1.592 | 3.5 | 10.0 |
| | 4 | 13 | 6.846 | 1.689 | 3.8 | 9.4 |
| Exposed | 1 | 18 | 6.533 | 1.731 | 3.9 | 10.9 |
| | 2 | 18 | 6.339 | 1.434 | 4.2 | 9.6 |
| | 3 | 18 | 6.756 | 1.490 | 3.6 | 9.7 |
| | 4 | 17 | 6.541 | 1.177 | 4.8 | 9.5 |
| **Red Blood Cell Count ($10^6$)** | | | | | | |
| Control | 1 | 18 | 5.082 | 0.519 | 4.17 | 6.24 |
| | 2 | 19 | 4.984 | 0.440 | 4.24 | 5.71 |
| | 3 | 19 | 4.877 | 0.366 | 4.27 | 5.52 |
| | 4 | 13 | 5.015 | 0.458 | 4.31 | 6.02 |
| Exposed | 1 | 18 | 5.087 | 0.510 | 4.16 | 6.06 |
| | 2 | 18 | 4.996 | 0.435 | 4.09 | 5.73 |
| | 3 | 18 | 4.977 | 0.531 | 3.88 | 5.68 |
| | 4 | 17 | 5.078 | 0.554 | 4.07 | 5.97 |
| **Hemoglobin(g/dL)** | | | | | | |
| Control | 1 | 18 | 15.789 | 1.458 | 13.1 | 18.9 |
| | 2 | 19 | 15.963 | 1.381 | 13.7 | 18.2 |
| | 3 | 19 | 15.632 | 1.215 | 13.6 | 17.7 |
| | 4 | 13 | 15.162 | 1.039 | 13.3 | 17.0 |
| Exposed | 1 | 18 | 15.289 | 1.405 | 12.2 | 17.3 |
| | 2 | 18 | 15.467 | 1.349 | 12.9 | 17.8 |
| | 3 | 18 | 15.461 | 1.471 | 12.8 | 17.4 |
| | 4 | 17 | 15.065 | 1.522 | 11.3 | 17.0 |
| **Hematocrit (%)** | | | | | | |
| Control | 1 | 18 | 46.167 | 3.946 | 39.3 | 54.1 |
| | 2 | 19 | 45.500 | 3.205 | 39.5 | 50.8 |
| | 3 | 19 | 45.095 | 2.785 | 40.7 | 50.5 |
| | 4 | 13 | 46.492 | 3.223 | 41.2 | 52.0 |
| Exposed | 1 | 18 | 44.650 | 4.037 | 36.3 | 50.9 |
| | 2 | 18 | 43.889 | 3.466 | 37.1 | 50.9 |
| | 3 | 18 | 44.344 | 3.995 | 36.5 | 49.9 |
| | 4 | 17 | 45.165 | 4.853 | 34.8 | 50.9 |

| Parameter | Period | # Samples | Mean | S.D. | Minimum | Maximum |
|---|---|---|---|---|---|---|
| **MCV (Mean Corpuscular Volume) (fL)** | | | | | | |
| Control | 1 | 18 | 91.028 | 2.997 | 86.7 | 97.5 |
| | 2 | 19 | 91.458 | 3.198 | 86.6 | 97.4 |
| | 3 | 19 | 92.384 | 3.260 | 86.1 | 98.0 |
| | 4 | 13 | 92.885 | 4.024 | 86.4 | 100.6 |
| Exposed | 1 | 18 | 87.933 | 3.387 | 81.8 | 94.0 |
| | 2 | 18 | 87.956 | 2.819 | 83.5 | 95.6 |
| | 3 | 18 | 89.289 | 3.176 | 83.4 | 96.4 |
| | 4 | 17 | 87.794 | 7.239 | 64.5 | 94.6 |
| **MCH (Mean Corpuscular Hemoglobin) (g/dL)** | | | | | | |
| Control | 1 | 18 | 31.083 | 0.926 | 29.3 | 32.8 |
| | 2 | 19 | 32.042 | 1.233 | 30.4 | 35.0 |
| | 3 | 19 | 31.989 | 1.149 | 29.9 | 34.8 |
| | 4 | 13 | 30.292 | 1.172 | 28.3 | 31.9 |
| Exposed | 1 | 18 | 30.111 | 1.218 | 28.5 | 33.0 |
| | 2 | 18 | 30.967 | 1.081 | 29.0 | 33.0 |
| | 3 | 18 | 31.178 | 1.208 | 29.0 | 33.3 |
| | 4 | 17 | 29.724 | 1.636 | 25.5 | 31.8 |
| **MCHC (Mean Corpuscular Hemoglobin Concentration) (g/dL)** | | | | | | |
| Control | 1 | 18 | 34.167 | 0.737 | 33.0 | 35.7 |
| | 2 | 19 | 35.042 | 0.794 | 34.0 | 36.3 |
| | 3 | 19 | 34.632 | 0.998 | 33.0 | 36.2 |
| | 4 | 13 | 32.608 | 0.731 | 31.2 | 33.8 |
| Exposed | 1 | 18 | 34.239 | 0.663 | 33.5 | 35.9 |
| | 2 | 18 | 35.228 | 0.735 | 33.7 | 36.7 |
| | 3 | 18 | 34.911 | 0.581 | 33.8 | 36.1 |
| | 4 | 17 | 33.376 | 0.747 | 32.2 | 34.7 |
| **Platelet Count ($10^3$)** | | | | | | |
| Control | 1 | 18 | 284.278 | 64.510 | 203 | 454 |
| | 2 | 19 | 270.368 | 59.637 | 202 | 442 |
| | 3 | 19 | 278.211 | 59.170 | 188 | 390 |
| | 4 | 13 | 249.462 | 35.813 | 186 | 320 |
| Exposed | 1 | 18 | 264.000 | 45.656 | 188 | 377 |
| | 2 | 18 | 251.444 | 39.497 | 191 | 349 |
| | 3 | 18 | 261.722 | 35.312 | 202 | 355 |
| | 4 | 17 | 252.824 | 50.534 | 186 | 376 |

| Parameter | Period | # Samples | Mean | S.D. | Minimum | Maximum |
|---|---|---|---|---|---|---|
| **Neutrophils (%)** | | | | | | |
| Control | 1 | 18 | 55.567 | 8.809 | 45.8 | 82.2 |
| | 2 | 19 | 55.358 | 6.765 | 41.6 | 70.4 |
| | 3 | 19 | 56.874 | 6.333 | 46.6 | 67.1 |
| | 4 | 13 | 57.846 | 11.874 | 38.3 | 86.4 |
| Exposed | 1 | 18 | 56.933 | 8.053 | 44.9 | 81.5 |
| | 2 | 18 | 56.833 | 6.499 | 46.2 | 75.6 |
| | 3 | 18 | 58.422 | 6.495 | 47.5 | 74.3 |
| | 4 | 17 | 57.712 | 7.316 | 47.0 | 77.3 |
| **Lymphocytes (%)** | | | | | | |
| Control | 1 | 18 | 32.800 | 7.707 | 10.8 | 44.3 |
| | 2 | 19 | 32.600 | 6.926 | 17.3 | 46.2 |
| | 3 | 19 | 31.432 | 6.885 | 20.5 | 44.5 |
| | 4 | 13 | 31.077 | 9.637 | 8.3 | 48.0 |
| Exposed | 1 | 18 | 31.706 | 7.079 | 12.0 | 45.3 |
| | 2 | 18 | 31.794 | 6.379 | 18.1 | 45.7 |
| | 3 | 18 | 30.522 | 5.868 | 18.8 | 41.6 |
| | 4 | 17 | 31.000 | 7.080 | 15.4 | 44.3 |
| **Monocytes (%)** | | | | | | |
| Control | 1 | 18 | 8.211 | 1.901 | 5.8 | 13.1 |
| | 2 | 19 | 8.379 | 1.698 | 5.1 | 11.3 |
| | 3 | 19 | 7.953 | 1.834 | 4.2 | 10.4 |
| | 4 | 13 | 7.946 | 2.437 | 4.0 | 11.8 |
| Exposed | 1 | 18 | 7.861 | 1.800 | 5.6 | 11.1 |
| | 2 | 18 | 7.822 | 1.341 | 5.7 | 10.6 |
| | 3 | 18 | 7.622 | 2.086 | 4.7 | 13.6 |
| | 4 | 17 | 7.935 | 1.393 | 6.0 | 11.1 |
| **Eosinophils (%)** | | | | | | |
| Control | 1 | 18 | 2.722 | 1.596 | 0.2 | 6.9 |
| | 2 | 19 | 2.958 | 1.953 | 0.8 | 9.0 |
| | 3 | 19 | 2.516 | 1.135 | 1.1 | 5.1 |
| | 4 | 13 | 2.400 | 1.601 | 0.4 | 6.1 |
| Exposed | 1 | 18 | 2.883 | 1.767 | 0.4 | 6.3 |
| | 2 | 18 | 2.856 | 1.489 | 0.1 | 5.5 |
| | 3 | 18 | 2.767 | 1.190 | 0.4 | 4.5 |
| | 4 | 17 | 2.394 | 1.144 | 0.5 | 5.0 |

| Parameter | Period | # Samples | Mean | S.D. | Minimum | Maximum |
|---|---|---|---|---|---|---|
| **Basophils (%)** | | | | | | |
| Control | 1 | 18 | 0.700 | 0.416 | 0.2 | 1.4 |
| | 2 | 19 | 0.705 | 0.590 | 0.0 | 2.3 |
| | 3 | 19 | 1.221 | 1.312 | 0.0 | 4.0 |
| | 4 | 13 | 0.731 | 0.601 | 0.1 | 2.0 |
| Exposed | 1 | 18 | 0.617 | 0.422 | 0.1 | 1.5 |
| | 2 | 18 | 0.689 | 0.519 | 0.0 | 1.8 |
| | 3 | 18 | 0.650 | 0.603 | 0.0 | 1.9 |
| | 4 | 17 | 0.959 | 1.062 | 0.0 | 4.1 |
| **Blood Urea Nitrogen (mg/kg)** | | | | | | |
| Control | 1 | 18 | 13.778 | 3.021 | 9 | 19 |
| | 2 | 19 | 13.158 | 2.949 | 8 | 19 |
| | 3 | 19 | 10.211 | 2.780 | 6 | 15 |
| | 4 | 13 | 13.000 | 2.160 | 9 | 17 |
| Exposed | 1 | 18 | 11.889 | 2.742 | 8 | 17 |
| | 2 | 18 | 11.389 | 2.973 | 6 | 17 |
| | 3 | 18 | 10.222 | 3.318 | 6 | 19 |
| | 4 | 17 | 12.176 | 3.557 | 6 | 18 |
| **Creatinine (mg/dL)** | | | | | | |
| Control | 1 | 18 | 1.039 | 0.146 | 0.7 | 1.3 |
| | 2 | 19 | 0.995 | 0.127 | 0.8 | 1.2 |
| | 3 | 19 | 0.963 | 0.121 | 0.7 | 1.1 |
| | 4 | 13 | 1.008 | 0.150 | 0.8 | 1.3 |
| Exposed | 1 | 18 | 1.028 | 0.136 | 0.8 | 1.3 |
| | 2 | 18 | 1.000 | 0.153 | 0.7 | 1.3 |
| | 3 | 18 | 1.072 | 0.336 | 0.8 | 2.2 |
| | 4 | 17 | 1.024 | 0.168 | 0.8 | 1.3 |
| **AST (Aspartamine Transferase) (IU/L)** | | | | | | |
| Control | 1 | 18 | 26.556 | 7.994 | 13 | 47 |
| | 2 | 19 | 29.316 | 10.350 | 14 | 57 |
| | 3 | 19 | 29.947 | 17.296 | 13 | 92 |
| | 4 | 13 | 27.000 | 7.800 | 14 | 45 |
| Exposed | 1 | 18 | 27.222 | 11.735 | 20 | 62 |
| | 2 | 18 | 27.167 | 9.109 | 16 | 56 |
| | 3 | 18 | 26.111 | 8.159 | 16 | 44 |
| | 4 | 17 | 28.235 | 9.223 | 18 | 58 |

| Parameter | Period | # Samples | Mean | S.D. | Minimum | Maximum |
|---|---|---|---|---|---|---|
| **ALT (Alanine Transferase)** | | | | | | |
| Control | 1 | 18 | 37.111 | 18.802 | 16 | 85 |
| | 2 | 19 | 33.105 | 25.921 | 14 | 130 |
| | 3 | 19 | 33.211 | 21.534 | 12 | 91 |
| | 4 | 13 | 33.462 | 15.360 | 19 | 72 |
| Exposed | 1 | 18 | 36.778 | 28.327 | 18 | 124 |
| | 2 | 18 | 30.500 | 23.861 | 13 | 116 |
| | 3 | 18 | 30.111 | 15.922 | 15 | 77 |
| | 4 | 17 | 37.059 | 18.236 | 18 | 88 |
| **Alkaline Phosphatase** | | | | | | |
| Control | 1 | 18 | 83.333 | 19.241 | 55 | 115 |
| | 2 | 19 | 76.474 | 20.508 | 42 | 121 |
| | 3 | 19 | 64.842 | 17.718 | 41 | 88 |
| | 4 | 13 | 81.462 | 29.624 | 49 | 163 |
| Exposed | 1 | 18 | 88.333 | 20.026 | 49 | 131 |
| | 2 | 18 | 78.389 | 15.515 | 40 | 104 |
| | 3 | 18 | 64.167 | 13.422 | 40 | 84 |
| | 4 | 17 | 82.294 | 15.503 | 50 | 107 |

Appendix C: Report of the Analysis of the Stress Proteins

## Two-dimensional Electrophoresis of Serum Proteins from JP4/JP8-exposed Humans

**Submitted by:** Frank A. Witzmann, Ph.D.
Professor of Biology
Molecular Anatomy Laboratory
Department of Biology
Indiana Univ. Purdue Univ. Columbus
Columbus, Indiana 47203

**Background.** Our laboratory has conducted extensive two-dimensional electrophoretic (2-DE) protein mapping studies characterizing the effects of numerous chemical toxicants in a variety of *in vivo* and *in vitro* models by quantitatively and qualitatively examining cellular protein expression. Through these efforts we have established 2-DE protein databases and applying this novel and robust approach to analyzing the effects of Air Force-relevant chemical exposure. Recently we have concentrated our efforts on the expression of stress proteins as biomarkers of chemical exposure and effect. This capability drew the attention of Air Force scientists at Hill AFB who wished to determine the effect of a regional switchover in jet fuel from JP-4 to JP-8 on AF personnel.

Our laboratory's planned involvement in this ongoing investigation was to generate 2-DE protein maps of human serum samples from control and exposed individuals and to analyze the resulting patterns for stress protein expression. Unfortunately, these classic "stress proteins" are typically intracellular proteins, many associated with membrane organelles, and thus absent from extracellular fluids like serum. However, because the human serum protein 2-DE pattern is well characterized, our intent was to examine the pattern for any detectable changes resulting from exposure, paying particular attention to the "Acute-Phase Proteins (APP)." This group of >30 proteins serve as serum markers of the acute phase response, a complex set of neurological, endocrine, and metabolic alterations that occur locally and systemically following injury, infection, immunologic reaction, and inflammation.

Although the APP were of principal interest, our 2-DE capabilities ensured detection of any statistically significant alterations in any of the >500 proteins we expected to resolve.

**Approach.** Serum samples were received from the following groups of various individuals: those who had been exposed to JP-4, JP-8 (3 mo.), JP-8 (6 mo.), JP-8 (18 mo.), and samples from the corresponding unexposed control group at each time point, for a total of 8 sample-groups. Serum samples were solubilized in an SDS-urea solubilization buffer. Sample proteins were separated by 2-DE using the 20 x 25 cm

ISO-DALT[7] 2D gel system operating with 20 gels per batch. All first dimension isoelectric focusing (IEF) gels were prepared using the same single standardized batch of ampholytes (BDH Resolyte 3.5-10) selected for ongoing 2-DE serum protein database work. Ten to twenty microliters of solubilized protein sample were applied to each gel and the gels were run for approximately 25,000 volt-hours using a progressively increasing voltage protocol implemented by a programmable high voltage power supply.

A computer-controlled gradient casting system was used to prepare second dimension SDS gradient slab gels in which the top 5% of the gel was 11%T acrylamide, and the lower 95% of the gel varied linearly from 11% to 17%T. Each gel was identified by a computer-printed filter paper label polymerized into the gel. First dimension IEF tube gels were loaded directly onto the slab gels without equilibration. Second dimension slab gels were run in groups of 20 in a DALT slab electrophoresis tank at $10^{N}C$. The runs lasted approximately 18 hr at 160V.

Following SDS electrophoresis, slab gels were stained for protein using a colloidal Coomassie Blue G-250 procedure in covered plastic boxes, with 10 gels per box. This procedure involved fixation in 1.5 liter of 50% ethanol/2% phosphoric acid overnight, three 30 minute washes in 2 liters of cold tap water, and transfer to 1.5 liters of 30% methanol/17% ammonium sulfate/3% phosphoric acid for one hour followed by addition of a gram of powdered Coomassie Blue G-250 stain. Staining required approximately 4 days to reach equilibrium intensity.

Each stained slab gel was digitized at 125 micron resolution, using an Ektron 1412 scanner and high-intensity light box. Raw, digitized gel images were archived on high-density DAT tape and a greyscale videoprint prepared from the raw digital image as hard-copy backup of the gel image. Each 2-DE gel was processed using the Kepler software system with procedure PROC008 which provided a spotlist giving position, shape and density information for each detected spot. This procedure made use of digital filtering, mathematical morphology techniques and digital masking to remove background, and used full two-Processing parameters and file locations were stored in a relational database, while various log files detailing operation of the automatic analysis software were archived with the reduced data.

The specific experiment package was constructed using the Kepler experiment definition database to assemble groups of 2-DE patterns corresponding to the various control and experimental groups associated with each experiment. Each pattern was matched to the appropriate "master" pattern, thereby providing future linkage to a human serum protein 2-DE database under development. The groups of gels making up an experiment were then scaled together (to eliminate quantitative differences due to gel loading or staining differences) by a linear procedure based on a selected set of spots. These spots were selected by the GOODSCALE procedure, which selects spots which are Gaussian converged, have a good initial intra-group coefficient of variation (CV), have a good (non-elongated) shape, an integrated density between certain limits

(avoiding very small or overloaded spots), and were detected on almost all gels of the set.

Groupwise statistical comparisons (Student's t-test) were made graphically and interactively and the results displayed in montage format using the KPL42 module. Graphical results of individual spot statistics and spot maps were printed in postscript while raw gel images and spot profiles were printed using a 64 level greyscale videoprinter.

**Results.** The figure below illustrates the digitized 2-DE human serum protein pattern displayed as a composite, representative of the serum samples in this study. Identifications made on this gel image are tentative, based on published patterns as well as those accessible on the Web.

In the imaging and 2D analysis system used in this experiment, the master pattern, to which all sample patterns are compared, is essentially a composite of all proteins resolved in all the various samples. It is used to match the protein spots appearing in all other sample patterns and is, therefore, not representative of any one sample pattern. The master pattern for human serum used in our laboratory for the running conditions described is shown below. By convention, acidic proteins are oriented to the left, basic to the right, low MW at the bottom, and high MW proteins toward the top of the pattern. Each circle or ellipse represents a single, detected protein. The horizontal dimension represents a pH gradient of approximately 4-8. In this particular example, the darkened circles and ellipses represent some of the tentatively identified acute phase proteins. An average of 537 serum proteins were resolved in the 8 sample groups studied. Of this total, approximately 350 spots were successfully matched in the sample patterns. While these data are quite comparable to similar studies of human serum protein 2-DE conducted in other laboratories, they affirm the heterogeneous nature of this population of proteins, and the inherent difficulty in managing sample variability in human sera.

To accurately and reproducibly evaluate chemical exposure effects, each step in the 2-DE technique is designed to minimize sample (and consequent protein pattern) variation due to fluctuations in procedure. To this end, chemicals used in every aspect of the approach (from tissue solubilization to gel staining and protein identification) are purchased in huge lots and huge volumes of stock solutions are prepared. Despite our best efforts, inherent sample variation tends to confound our assessment of chemically-induced alterations. A statistical measure of this variability typically used in our experiments is the coefficient of variation or CV. The coefficient of variation is a measure of relative dispersion, is given by

Coefficient of Variation = Standard Deviation / Mean

and is generally expressed as a percentage. The use of coefficient of variation lies partly in the fact that the mean and standard deviation tend to change together in many experiments. A knowledge of relative variation is valuable in evaluating the consistency of protein resolution In previous experiments, we analyzed the CV in both *in vivo* studies and data from *in vitro* studies (tissue slices). These data are shown in the following table. Of the 1000 proteins resolved in each type of sample (including studies in which toxicant exposures were conducted), the number of proteins with CV<15% the following are observed:

| Sample Type | Total # of Protein Spots Resolved | # of Protein Spots with CV<15% | % of Protein Spots with CV<15% |
|---|---|---|---|
| *In vivo* (fresh rat liver, 7 dose groups) | 1469 | 501 | 34% |
| *In vivo* (preserved human liver, 1 group) | 1005 | 183 | 18% |
| *In vitro* (bovine testis slice, 5 dose groups) | 979 | 372 | 38% |
| *In vivo* (human serum, 8 groups) | 537 | 11 | 2.1% |

These data clearly demonstrate the heterogeneity of the human serum samples studied. Given the sample variation, it is not surprising that neither the paired nor unpaired t-tests of the protein abundance (integrated spot density) showed any significant differences related to jet fuel exposure. Despite the variation, we are quite satisfied with the resolution and spot detection observed. The data presented here suggest that any alterations in serum protein patterns associated with conversion from JP-4 to JP-8 exposure are undetectable by the 2-DE methods used.

**Recommendations:**

We have very recently analyzed serum and tissue samples from Swiss-Webster mice exposed to JP-8 jet fuel by particulate inhalation studies conducted by Dr. Mark Witten at the University of Arizona. Preliminary results indicate not only significant tissue effects, but changes in the 2-DE pattern of serum protein expression as a result of JP-8 inhalation. A homogeneous population of exposed animals may be a better initial indicator of the hazards of JP-8 exposure than a group of variant humans.

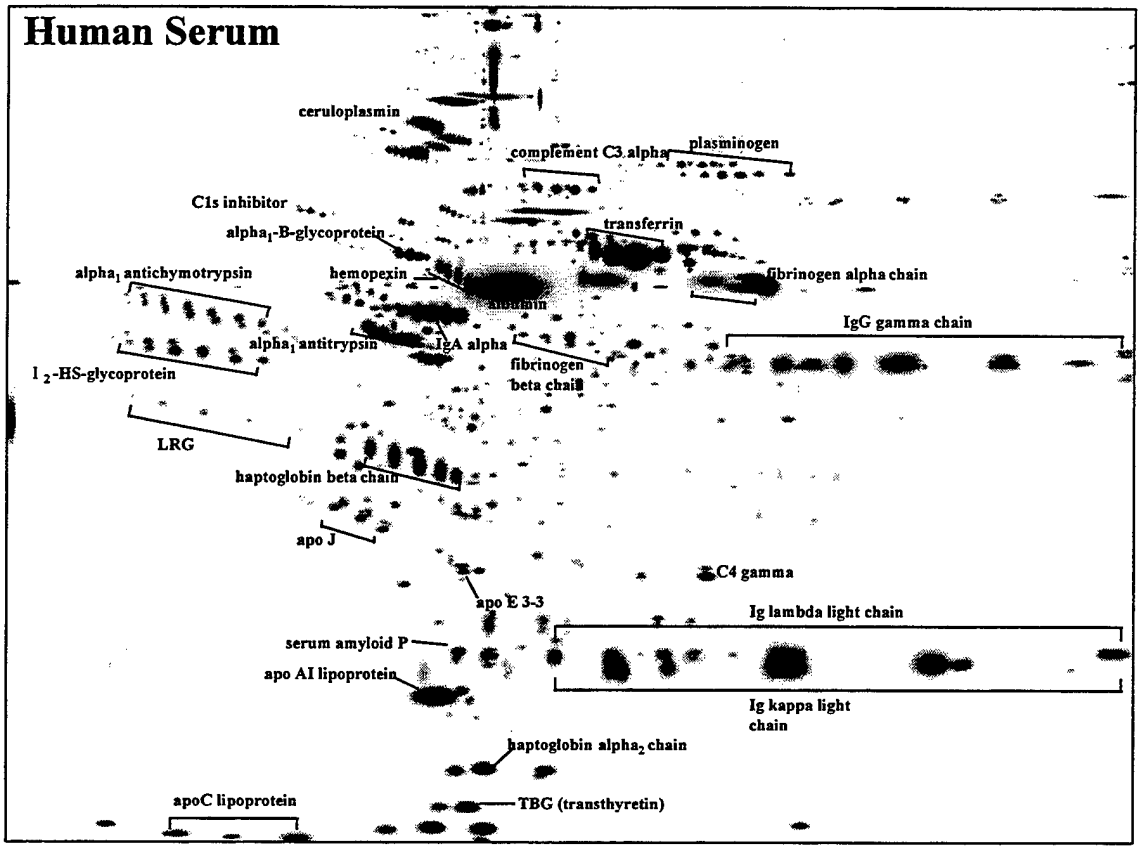


Figure C-1. Digitized 2-DE human serum protein patterns.

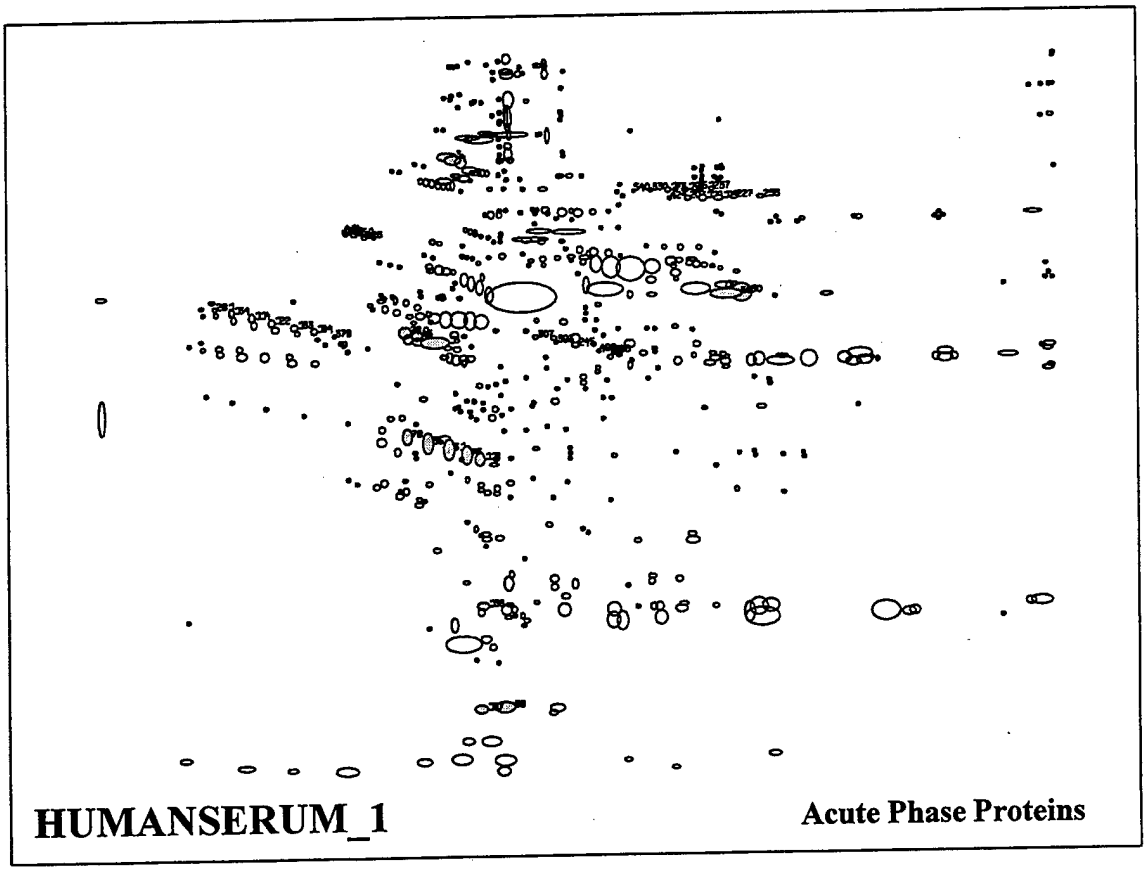


Figure C-2: Acute Phase Proteins